J. Dairy Sci. 106:6951–6960
https://doi.org/10.3168/jds.2022-22969
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Expression of enzymes involved in the urea cycle and muscle and mammary gland development of Holstein × Gyr heifers in a rotational grazing system supplemented with increasing protein levels

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ABSTRACT

Studies evaluating the crude protein (CP) supplementation strategies across the year for grazing cattle and its association with the enzymes involved in the urea cycle and muscle and mammary gland developments are scarce. Thus, we aimed to evaluate the effect of supplementation with different levels of CP on the expression of genes involved in the urea cycle and muscle and mammary gland development of Holstein × Gyr crossbreed heifers grazing intensively managed *Brachiaria decumbens* throughout the year. Thirty-eight heifers with average initial BW of 172.5 ± 11.15 kg (mean ± SE) and 8.2 ± 0.54 mo of age were randomly assigned to 1 of 4 treatments: 3 protein supplements (SUP) fed at 5g/kg of body weight, plus a control group (CON, non-supplemented animals). The supplement CP levels evaluated were: 12, 24, and 36%. The study was divided into 4 seasons: rainy, dry, rainy-dry transition (RDT), and dry-rainy transition (DRT). On the penultimate day of each season, ultrasound images of the carcass and mammary gland were taken. Five animals from each treatment were randomly chosen on the last day of each season, and liver and muscle tissue biopsies were performed. The target genes were the mammalian target of rapamycin (mTOR) and adenosine monophosphate-activated protein kinase (AMPK) in the muscle samples. Carbamoyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), arginosuccinate lyase (ASL), and arginase (ARG) were evaluated in the liver samples. Data were analyzed using PROC GLIMMIX of the SAS with repeated measures. We observed a greater rib eye area (cm^2) and fat thickness (mm) in SUP animals than in non-supplemented animals. However, we did not observe differences among SUP levels for both variables. No effects of supplementation were detected on mammary gland development. Nevertheless, seasonal effects were observed, where the RDT and dry season had the most and least accumulated fat in the mammary gland. In muscle, we observed greater expression of AMPK in non-supplemented animals than SUP animals. On the other hand, no differences were observed in gene expression between SUP and non-supplemented animals and among SUP animals for mTOR. Season affected both AMPK and mTOR; heifers had a greater AMPK gene expression on rainy than RDT. For mTOR, we observed greater gene expression in RDT and DRT than in rainy. No differences were observed among RDT, dry, and DRT, and between dry and rainy seasons for mTOR. We observed greater CPS, ASL, and ARG gene expression in SUP animals than in non-supplemented animals. Among SUP animals, supplement CP linearly affected CPS. In conclusion, the supplementation strategy did not affect mammary gland development and mTOR expression in muscle tissue. However, we observed a seasonal effect on mammary gland development and AMPK and mTOR expression. The CP supplementation increased the rib eye area and fat thickness, directly affecting AMPK expression in the muscle. Moreover, the CP supplementation increased urea cycle enzyme expression, indicating greater urea production in the liver.
Key words: biopsy, liver, nitrogen metabolism, pasture

INTRODUCTION

Crude protein supplementation strategies for grazing cattle are a constant research focus because exclusive pasture rarely provides a balanced diet, which limits animal performance (Detmann et al., 2014a; Batista et al., 2016; da Silva-Marques et al., 2018). Furthermore, pasture varies qualitatively and quantitatively throughout the year, where during the rainy season, tropical forages have higher quality (Detmann et al., 2014b), and during the dry season, the pasture quality

Received October 31, 2022.
Accepted March 24, 2023.
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drops because of its high fiber and low CP contents (Sampaio et al., 2010). Thus, understanding nitrogen (N) metabolism and its connection with pasture composition throughout the year is necessary to plan the best supplementation strategy for grazing animals. Different supplementation strategies in each season will be essential to improve animal performance and efficiency of N use.

Under normal nutritional conditions, ammonia absorbed from the rumen is transported to the liver, entering the ornithine cycle, and is converted into urea. This urea can be recycled via the rumen wall, reused as a nitrogen source for microbial protein or excreted in the urine when CP is in excess (Lapierre and Lobley, 2001). Thus, excess urea excreted in the urine is one of the main factors contributing to the low efficiency of N use, which negatively affects the environment (Dijkstra et al., 2013).

The process of converting ammonia into urea in the ornithine cycle is performed in 5 steps by 5 key enzymes (Takagi et al., 2008). First, the cycle needs carbamoyl phosphate synthetase (**CPS**) and ornithine transcarbamylase (**OTC**), located in the mitochondrial matrix; then, the cycle needs argininosuccinate synthetase (**ASS**), arginosuccinate lyase (**ASL**), and arginase (**ARG**), located in the cytosol (Takiguchi and Mori, 1995). Despite the crucial importance of these 5 enzymes in the urea cycle, the literature lacks studies evaluating their expression and association with supplementation strategy and pasture quality across the year.

In general, CP supplementation promotes greater performance and, consequently, high body gain and fat deposition rates in grazing cattle (Guerra et al., 2016; Machado et al., 2019; Machado et al., 2020). On the other hand, previous studies have demonstrated that dairy heifers submitted to a high-performance diet without an adequate nutritional balance between energy and protein may impair the development of the mammary gland due to excessive fat deposition (Weller et al., 2016; Albino et al., 2017b). However, limited studies evaluate CP supplementation strategies while monitoring body and mammary gland development in grazing dairy heifers.

We hypothesized that the expression of the genes involved in the urea cycle might be affected by the interaction between CP supplementation strategy and the season of the year for grazing dairy heifers, where high CP supplement levels would promote a greater expression of genes involved in the urea cycle, mainly during seasons where pasture has high CP content. Moreover, we predicted that CP supplementation would promote a greater activity of genes involved in tissue synthesis, affecting muscle and mammary gland lean tissue gain. Therefore, we aimed to evaluate the effect of increasing CP supplementation on the genes expression of enzymes involved in the urea cycle and muscle tissue growth, mammary gland, and muscle development of Holstein × Gyr crossbreed heifers grazing intensively managed *Brachiaria decumbens* throughout the year.

MATERIALS AND METHODS

The experiment was conducted in the Department of Animal Science of the Universidade Federal de Viçosa (Viçosa, Minas Gerais, Brazil; 20°45′ S and 42°52′). All animal handling and procedures were approved by the Ethics Committee for Animal Use at Universidade Federal de Viçosa, under protocol no. 041/2017. More information regarding this study's management practices can be accessed in Castro (2023).

Treatments and Experimental Design

Thirty-eight crossbred heifers (1/2 Holstein × 1/2 Gyr), with average initial BW of 172.5 ± 11.15 kg (mean ± SE) and 8.2 ± 0.54 mo of age, were used. Initially, all heifers were treated for ectoparasites and endoparasites (Ivomec, Paulina, Sao Paulo, Brazil). Then, the heifers were randomly assigned to 1 of 4 treatments using the random function in Excel: 3 supplements (**SUP**) had increasing levels of CP composed of soybean meal and corn ground, plus a control group (**CON**; n = 10) in which the animals received only mineral mixture ad libitum. The supplements had 12 (90.96% of ground corn and 9.04 of soybean meal), 24 (59.32% of ground corn and 40.68% of soybean meal), and 36% (27.68% of ground corn and 72.32 of soybean meal) of CP for treatments S12 (n = 9), S24 (n = 10), and S36 (n = 9), respectively, respectively and fed at 5 g/kg of BW.

The supplements had 12, 24, and 36% of CP for treatments S12 (n = 9), S24 (n = 10), and S36 (n = 9), respectively, respectively and fed at 5 g/kg of BW.

The experiment lasted from January 7 (2018) to January 22 (2019). The experiment was divided into 4 seasons, plus an adaptation period of 30 d. The adaptation period lasted from January 7 to February 6, during which all heifers were fed the same supplement containing 18% CP at 5 g/kg of BW. The first season lasted from February 7 to April 24 (76 d), named the rainy-dry transition (**RDT**) season. The second season, named the dry season, lasted from April 25 to July 27 (94 d). The third season lasted from July 28 to November 5 (101 d) and was named the dry-rainy transition (**DRT**) season. Finally, the fourth season lasted from November 6 to January 22 (78 d) and was named the rainy season.

The heifers were managed in a rotational grazing system, with 27 paddocks (1700 m^2 each) of *Brachi-*

aria decumbens fertilized with 120 kg of N and 60 kg of K_2O per hectare per year. An additional area on the side of the main area was used during the dry season, with 23 paddocks (1700 m^2 each). All paddocks had free access to a resting area with shade (3 m^2/animal), water, and ad libitum mineral mix. All heifers were kept in the same paddock, accessing one paddock per day, except during the dry season, when accessed 2 paddocks per day (using the additional area described above) due to low pasture DM production in that season.

Every day, at 1000 h, all heifers were moved from the pasture to a management area (250 m distance), and supplements were fed to each group (treatment) separately. However, we estimated the individual supplement intake using TiO_2 as the external marker, following Titgemeyer et al. (2001) methodology. Thus, the experimental unit was the animal. The supplement was supplied at 5 g/kg of BW per day with a feeder bunk space of 50 cm per animal, and no orts were observed. The amount of supplement (5g/kg of BW) was chosen to meet the energy requirements of crossbreed dairy heifers, with an ADG of 0.5 kg/d grazing *Brachiaria decumbens* according to recommendations of NRC (2001). All heifers were weighed every 15 d to adjust the supplement supply. The animals of the control treatment (non-supplemented) were also moved to the management area, but no supplementation was provided. We spent 1 h with this management daily, and the animals returned to paddocks afterward.

The last 14 d of each season were used to collect samples (collection period) of pasture, feces, urine, body measures, weighing animals, liver and muscle tissue biopsy, and blood samples. Days 1 to 8 of the collection period were used to estimate digestibility, pasture intake, and supplemented intake. From d 9 to 11, we took body measurements and weighed the animals. On the 12th d, the carcass and mammary gland ultrasound images were taken. On d 13, liver and muscle tissue biopsies were performed, and on d 14, blood samples were collected. Information regarding analysis and results of intake, digestibility, performance, and blood paraments can be observed in Castro (2023). This study focused on ultrasound analyses of carcass and mammary gland development and liver and muscle biopsies.

Carcass Characteristics and Mammary Gland Development

Ultrasound images of the carcass and the mammary gland were taken on d 0 of the experiment and d 12 of each collection period. For the carcass characteristics, measurements of the *gluteus medius* and the *biceps femoris* muscle intercessions were taken by scanning between the 12th and 13th ribs and the rump in the P8 region. We used an 18-cm linear array ultrasound instrument (Aloka SSD-500V, Aloka Co. Ltd., Tokyo, Japan) operated at a frequency of 3.5 MHz. A standoff (Aloka long standoff guide-beef, Aloka Co. Ltd. Tokyo, Japan) and vegetable oil were used for adequate acoustic contact between the transducer, the standoff, and the animals' skin. Ultrasound images were recorded and later analyzed for backfat thickness and loin depth using the BioSoft Toolbox II for 200 Beef (Biotronics Inc., Ames, Iowa, USA) software.

Mammary gland ultrasound images were taken using a micro convex transducer (Mindray DP2200, Shenzhen, China), operating at 6 MHz (Albino et al., 2017a). Images of each mammary quarter were taken in a standardized position, with an inclination of 45° in relation to teat insertion, and recorded in bitmap format, a technique described by Albino et al. (2017a). Mammary gland ultrasound images were evaluated for pixel values in an 8-bit format as defined by Albino et al. (2017a) using ImageJ software (2011; National Institutes of Health, Bethesda, MD). The pixel value was estimated according to the brightness on a scale of 256 shades of gray (0 = black and 256 = white). Before the analysis, the software was calibrated for 100 pixels/cm pixel scale using the straight tracer tool. The pixel value of each mammary quarter was obtained as the mean from 3 squares (16 mm^2 each) randomly collected near the ductal structures from each image of each quarter. Subsequently, the pixel value of the whole mammary gland was obtained as an average value of each mammary quarter.

Liver and Muscle Tissue Collection

Five animals from each treatment were randomly chosen for gene expression analysis in the muscle and liver tissue, and collections were performed at the end of each season, as mentioned above. The same animals were used for these analyses in all seasons.

The liver tissue samples were taken via needle biopsy (Tru-Cut biopsy needle; Care Fusion Corporation, San Diego, CA, USA) 4 h before the supplement was fed, following the procedure described by Mølgaard et al. (2012). An incision was performed between the 11th and 12th ribs from the right hepatic lobe following the procedure described by Miranda et al. (2010). Muscle tissue samples were made on the left side at the 13th rib, at 3-fifths of the distance from the medial to the lateral edge of the *Longissimus dorsi* muscle. After collection, liver (110 mg of tissue) and muscle (1.50 cm^3) samples were placed into cryotubes, frozen, and stored in liquid nitrogen at −196°C until processing and further analyses.

Table 1. Gene names, accession numbers, and primer sequences

Gene symbol[1]	Accession number	Primer sequence (5′–3′)
mTOR	XM_002694043.6	Forward:AAGGAGAAGGAACGGACA
		Reverse:CCAGCACACGAGGTAAATAG
AMPK	NM_001109802.2	Forward:AGTTGCCTACCACCTCAT
		Reverse:GTGGTGATCGTCGAGAAAC
CPS	I3750D07	Forward:CTGACTGACCCTTCCTACA
	I3751A07	Reverse:CCGAACTCATCCACTTCATC
OTC	I3750D08	Forward:AATGGGCTGTCGGATTTG
	I3750D09	Reverse:GGAGTGGAGGATGTTATTCC
ASS	I3750D10	Forward:TTCAGGGGCCAGGTGTA
	I3750D11	Reverse:ATCAACCGGCTCGTAGT
ASL	I3751A03	Forward:ACTGGTGTCATCTCTACCC
	I3751A04	Reverse:TTTCGGACCAGGTAGTAGG
ARG	I3751A05	Forward:GGTGGCAGAAGTCAAGAAG
	I3751A06	Reverse:CACCCAAATGACACAGAGG
18S	NR_036642.1	Forward:GCCGCTAGAGGTGAAATTCT
		Reverse:TCGGAACTACGACGGTATCT

[1]*mTOR* = mammalian target of rapamycin; *AMPK* = adenosine monophosphate-activated protein kinase; *CPS* = carbamoyl phosphate synthetase;*OTC* = ornithine transcarbamylase; *ASS* = argininosuccinate synthetase; *ASL* = arginosuccinate lyase; *ARG* = arginase; *18S* = ribosomal RNA. Accession number in GenBank (http://www.ncbi.nlm.nih.gov).

Gene Expression Analyses

The muscle and liver tissue samples were used for gene expression analysis. First, the total RNA was extracted from 0.1 g of tissue using Trizol (InvitrogenTM, Thermo Fisher Scientific, Oregon, USA), following the manufacturer's recommendations. Total RNA was quantified with a NanoDrop spectrophotometer, and the integrity was assessed in a 1% agarose gel. Next, the RNA samples were reverse transcribed into cDNA using the Cells-To-cDNA kit (Ambion). The quantitation of cDNA concentration was performed using 1 μL of sample (muscle or liver) in a NanoVue Plus spectrophotometer (GE Healthcare). Lastly, the samples were diluted to 10 ng/μL concentration, and the material was stored at −20°C for real-time PCR analysis.

Relative quantification real-time PCR (qRT-PCR) was performed in duplicate on an ABI Prism 7300 Sequence Detection System thermocycler (Applied Biosystems, Foster City, CA) using GoTaq qPCR Master Mix (Promega Corp.) following the manufacturer's instructions.The amplification efficiency of each gene was calculated by constructing a cDNA serial dilution curve at concentrations of 25, 75, and 225 ng cDNA and concentrations of 100, 200, and 400 ng of primer per reaction. The reactions were considered efficient when the amplification efficiency of the target gene and the control gene were approximately equal, with a tolerance of 5% variation in relation to the control gene. Amplification conditions for all systems were performed with an initial step at 95°C for 2 min, followed by the second step of 40 denaturation cycles at 95°C for 15 s, and a final extension at 60°C for 60 s.

The expression for each target gene for each heifer was determined by subtracting the cycle threshold (**Ct**) value for the geometric mean of the control genes from the target gene Ct (target gene Ct − Ct endogenous reference), where Ct reflects the PCR cycle number at which the fluorescence generated crosses an arbitrary threshold. Results are expressed relative to 18 S, using the $2^{-\Delta\Delta Ct}$ method, where the ΔΔCt was calculated as follows: (Ct, Target − Ct, 18S) treatment (**TREAT**) − (Ct, Target − Ct, 18S) control (**CONT**; Livak and Schmittgen, 2001). The primer pairs for each target and internal control gene are listed in Table 1.

Target genes evaluated in the current study were the mechanistic target of rapamycin (mTOR) and adenosine monophosphate-activated protein kinase (AMPK) in muscle tissue. The genes associated with the urea cycle in the liver were: carbamoyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), arginosuccinate lyase (ASL), and arginase (ARG).

Statistical Analysis

The response variables were analyzed using the PROC GLIMMIX procedure of SAS (version 9.4). For all variables, measurements at d 0 (ultrasound carcass and mammary gland) were tested as covariates and removed from the models as they were all non-significant ($P > 0.05$). The variables were analyzed as a completely randomized design, with the season included as a repeated measure in the model as follows:

$$Y_{ijk} = \mu + T_i + \delta_{ij} + S_k + (T \times S)_{ik} + \varepsilon_{ijk},$$

where Y_{ijk} = observation ijk; μ = the overall mean; T_i = fixed effect of treatment i; δ_{ij} = random error with mean 0 and variance σ^2_δ, the variance between animals within the treatment, equal to the covariance between repeated measurements within animals; S_k = fixed effect of season k; $T \times S_{ik}$ = fixed effect of interaction between treatment i and season k; and ε_{ijk} = random error with the mean 0 and variance σ^2, the variance between measurements within animals. Fifteen variance-covariance structures were tested for each response variable. Thus, we used the variance-covariance structure that provided the best fit based on the lower Akaike information criterion. The variance components were the most common variance-covariance structure used. Observations with externally studentized residuals higher than |2.5| were first checked to ensure they were not a recording error. After checking and evaluating that the data has no recording errors, these observations were considered outliers and consequently excluded from the data set. The analysis of possible outliers was performed only once for each outcome variable so as not to create erroneous results. In the end, we observed only one animal as an outlier. Least squares means were considered different when $P \leq 0.05$, and tendency was declared when $0.05 < P < 0.100$.

The pixel values from the analysis of ultrasound images did not follow a normal distribution, and we had to use logarithmic transformation to normalize the data for analysis. However, the pixel data results are shown in an untransformed format to make visualization easier.

All means were compared by the following orthogonal contrasts: (1) effect of supplementation (non-supplemented animals, CON, vs. supplemented animals, SUP); (2) linear effect of supplement CP level in supplemented animals; and (3) quadratic effect of supplement CP level in supplemented animals. In addition, those same contrasts were evaluated within each season in case of significant interactions. Moreover, season means were compared by the least squares means method, and differences were considered significant when $P < 0.05$ according to the Tukey test.

RESULTS

We observed a greater rib eye area (cm^2) in SUP animals than in non-supplemented animals ($P = 0.001$, Figure 1). However, we did not observe differences among SUP animals ($P = 0.166$). When we evaluated the effect of season, the greatest rib eye area was observed in the rainy season, followed by DRT, dry, and DRT ($P = 0.001$; Figure 1).

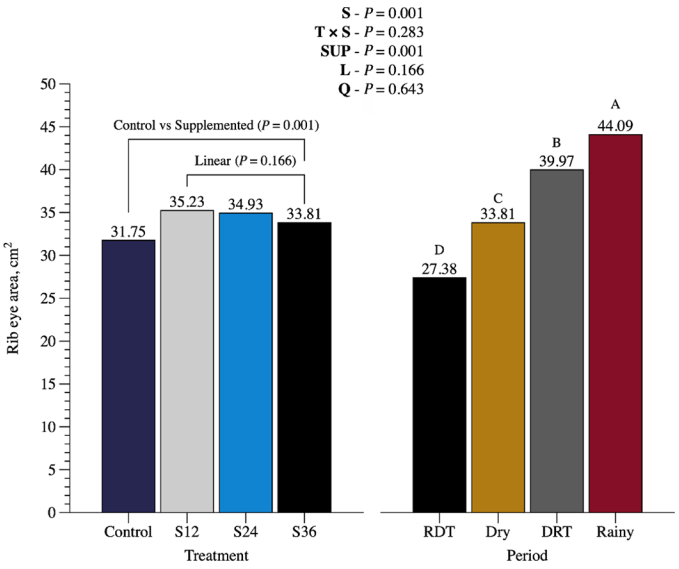

Figure 1. Rib eye area (cm^2) of crossbred Holstein × Gyr heifers nonsupplemented (Control) or supplemented with 12 (S12), 24 (S24), or 36% (S36) CP in supplement throughout experimental seasons: rainy-dry transition (RDT), dry, dry-rainy transition (DRT), and rainy. S = season; T × S = interaction between treatment and season; SUP = supplemented vs. non-supplemented; L = linear effect among supplemented animals, and Q = quadratic effect among supplemented animals. Letters A–D indicate differences among periods ($P < 0.05$).

We observed greater fat thickness (mm) in SUP animals than in non-supplemented animals ($P = 0.009$; Figure 2). Among SUP animals, no differences were detected ($P = 0.442$). However, a seasonal effect was detected ($P = 0.001$); in the rainy season, heifers had greater fat thickness (mm) than in other seasons ($P = 0.001$, Figure 2).

Treatment did not affect mammary gland development ($P > 0.267$; Figure 3). However, seasonal effects were observed; in the RDT and dry season, heifers had the greatest and lowest amount of fat accumulated in the mammary gland, respectively (Figure 3).

The results of relative mRNA abundance of AMPK and mTOR in muscle tissue and CPS, OTC, ASS, ASC, and ARG in liver tissue from crossbred Holstein × Gyr heifers supplemented with increasing supplement CP levels are shown in Table 2. In the muscle, we observed greater gene expression of AMPK in non-supplemented animals than in SUP animals ($P = 0.002$; Table 2). No differences were observed among SUP animals for AMPK ($P > 0.186$; Table 2). We detected no differences in gene expression between SUP and non-supplemented animals and among SUP animals for mTOR ($P > 0.305$; Table 2). However, seasonal effects were observed for AMPK and mTOR, as demonstrated in Table 3. For AMPK, a difference was observed between

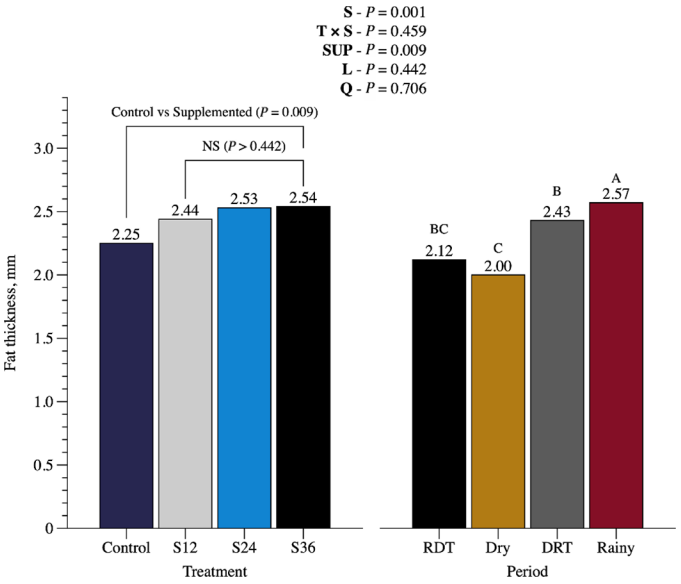

Figure 2. Fat thickness (mm) of crossbred Holstein × Gyr heifers nonsupplemented (Control) or supplemented with 12 (S12), 24 (S24), or 36% (S36) CP in supplement throughout experimental seasons: rainy-dry transition (RDT), dry, dry-rainy transition (DRT) and rainy. S = season; T × S = interaction between treatment and season; SUP = supplemented vs. non-supplemented; L = linear effect among supplemented animals, and Q = quadratic effect among supplemented animals. Letters A–C indicate differences among periods ($P < 0.05$).

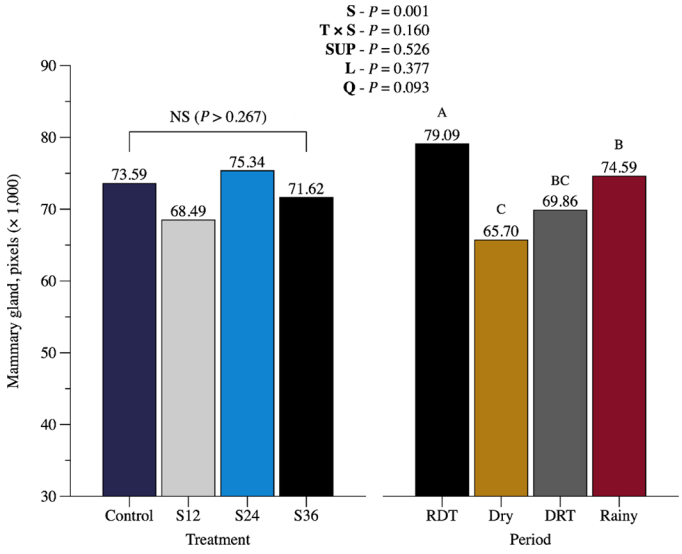

Figure 3. Mammary gland pixel patterns of crossbred Holstein × Gyr heifers nonsupplemented (Control) or supplemented with 12 (S12), 24 (S24), or 36% (S36) CP in supplement throughout experimental seasons: rainy-dry transition (RDT), dry, dry-rainy transition (DRT) and rainy. S = season; T × S = interaction between treatment and season; SUP = supplemented vs. non-supplemented; L = linear effect among supplemented animals, and Q = quadratic effect among supplemented animals. Letters A–C indicate differences among periods ($P < 0.05$).

the RDT and rainy seasons, whereby heifers had greater gene expression of AMPK in the rainy season compared with the RDT ($P = 0.05$; Table 3). No differences were observed for AMPK expression among RDT, dry and DRT, and among the dry, DRT and rainy ($P > 0.050$, Table 3). For mTOR, we observed greater gene expression of mTOR in the RDT and DRT seasons than in the rainy season ($P = 0.002$; Table 3). No differences were observed for mTOR expression among the RDT, dry and DRT seasons, and among the dry, and rainy seasons ($P > 0.050$, Table 3).

In the liver, we observed greater gene expression of CPS, ASL, and ARG in SUP animals than in non-supplemented animals ($P < 0.028$; Table 2). On the other hand, we detected no differences in gene expression between the SUP and non-supplemented animals for OTC and ASS ($P > 0.256$; Table 2). Furthermore, among SUP animals, differences were observed only for

Table 2. Gene expression of AMPK and mTOR in muscle tissue, and CPS, OTC, ASS, ASC, and ARG in liver tissue from crossbred Holstein × Gyr heifers supplemented with different crude protein levels in supplement throughout the experiment season

	Treatment[2]					*P*-value[3]				
Item[1]	CON	S12	S24	S36	SEM	S	T × S	SUP	L	Q
AMPK	0.055	0.041	0.047	0.039	0.004	0.050	0.764	0.002	0.664	0.186
mTOR	0.024	0.028	0.025	0.028	0.002	0.002	0.974	0.438	0.938	0.305
CPS	0.005	0.006	0.012	0.014	0.003	0.726	0.114	0.028	0.022	0.403
OTC	0.166	0.138	0.124	0.156	0.017	0.918	0.349	0.256	0.526	0.337
ASS	0.574	0.739	0.616	0.590	0.157	0.921	0.312	0.661	0.484	0.819
ASL	1.715	7.004	8.134	5.124	1.794	0.562	0.082	<0.001	0.399	0.355
ARG	15.789	59.096	82.680	25.940	19.271	0.424	0.056	0.004	0.088	0.081

[1]mTOR = mammalian target of rapamycin; AMPK = adenosine monophosphate activated protein kinase; CPS = carbamoyl phosphate synthetase; OTC = ornithine transcarbamylase; ASS = argininosuccinate synthetase; ASL = arginosuccinate lyase; ARG = arginase.

[2]CON = control; S12 = supplement with 12% CP; S24 = supplement with 24% CP; S36 = supplement with 36% CP.

[3]S = season effect; T × S = interaction between season and treatment; SUP = supplemented vs. nonsupplemented; L = linear effect among supplemented animals; Q = quadratic effect among supplemented animals.

Table 3. Gene expression of adenosine monophosphate-activated protein kinase (AMPK) and mechanistic target of rapamycin (mTOR) in muscle tissue in LSM $\pm$ $2^{-\Delta\Delta Ct}$ throughout the experiment season

	Season[1]					
Item	RDT	Dry	DRT	Rainy	SEM	*P*-value
AMPK	0.038[B]	0.045[AB]	0.043[AB]	0.055[A]	0.004	0.050
Mtor	0.030[A]	0.024[AB]	0.032[A]	0.021[B]	0.002	0.002

[A,B]Values followed by different letters in the row are different.

[1]RDT = rainy dry transition; DRT = dry rainy transition.

CPS, in which we detected a positive linear response ($P = 0.022$; Table 2). In other words, as the level of CP supplementation increased, the expression of CPS increased. In addition, no seasonal effects were observed for all enzymes evaluated in liver tissue ($P > 0.424$; Table 2), which were: CPS, OTC, ASS, ASL, and ARG.

DISCUSSION

An adequate CP supplementation strategy is necessary to increase animal performance and production efficiency; however, in several studies, researchers have associated ADG and balance between energy and protein with mammary gland development (Sejrsen and Purup, 1997; Albino et al., 2015). In this study, we did not observe any effect of the CP supplementation strategy on parenchymal mammary tissue growth; nevertheless, we observed that the mammary gland could remodel itself across seasons as we detected an evident season effect. The season effect was mainly due to the availability of nutrients. During the RDT season, the pasture had greater forage availability, as demonstrated in our companion study (Castro, 2023) and, consequently, a greater amount of fat was deposited in the mammary gland. On the other hand, pasture availability decreased quantitatively and qualitatively during the dry season, reducing heifer performance and fat in the mammary gland. In other words, the mammary gland can remodel itself across the year depending on performance and nutrient availability/intake in each season, accumulating more or less fat in the mammary gland. In agreement with these results, Albino et al. (2015) observed that parenchymal mammary tissues could be influenced by ADG and different proportions of the metabolizable energy and protein intake. Interestingly, in our study, a greater amount of fat in the mammary gland was observed during RDT season, followed by the rainy and dry seasons, following the same sequence of animal performance across seasons, in agreement with Albino et al. (2015).

The AMPK is a sensor of peripheral energy balance, and its activation is critical for maintaining energy balance in the body. In this sense, when cellular energy is low, AMPK is activated, and the processes that spend ATP are inhibited, such as protein synthesis (Appuhamy et al., 2014). Moreover, when AMPK is activated, we detected an increase in ATP production processes such as fatty acid oxidation and glucose uptake (Mukherjee et al., 2008). Conversely, the mTOR is responsible for increasing cell energy availability; thus, it could be seen as a sensor for nutrient availability, stimulating the body's anabolic processes and protein proliferation (Lie et al., 2019). As such, these pathways have been reported as the main drivers regulating energy balance in the muscle (Smith et al., 2013). In the present study, we detected a greater AMPK expression in non-supplemented animals than in SUP, which might indicate that the central AMPK signal pathway was inhibited in SUP animals, which promoted more significant lipid and muscle synthesis in these animals. As observed in our companion paper, these results are confirmed by greater intake, performance, IGF-1, and glucose levels in the SUP animals (Castro, 2023).

These results could also be confirmed by analyzing the rib eye area and fat thickness because the rib eye area indicates carcass muscularity and meat yield (Scholz et al., 2015). In fact, we observed a greater rib eye area and fat thickness in SUP animals than in non-supplemented animals. Furthermore, similar to our results, Underwood et al. (2008) observed lower AMPK activity in animals with high intramuscular fat, confirming that the activation of AMPK inhibits adipogenesis (Giri et al., 2006). Thereby, AMPK activity directly interferes with cellular proliferation, depending on its activation or not. Hence, the lower expression of AMPK in the SUP animals is likely linked to the greater rib eye area and fat thickness observed in these animals (Figure 3).

On the other hand, these differences were insufficient to support the same response in mTOR expression, where no differences were detected between SUP and non-supplemented animals and among SUP animals. Nevertheless, researchers have previously reported a negative association between mTOR and AMPK (Appuhamy et al., 2014) because they are regulators of cell growth, which is regulated by the availability of

nutrients (Lie et al., 2019). Curiously we observed a seasonal effect in AMPK and mTOR (Table 3) gene expression. Briefly, the greatest expression of AMPK and the lowest expression of mTOR were detected during the rainy season. These results are consistent with Appuhamy et al. (2014), who reported an inverse relationship between mTOR and AMPK phosphorylation.

Ruminants can recycle urea in the liver, which helps prevent excess N from becoming toxic to the animal. Urea recycled in the liver is released into the blood and can be excreted in the urine or re-enter the digestive tract by diffusion in saliva or across the rumen wall (Huntington and Archibeque, 2000). The urea cycle can be divided into 5 steps, and 5 different enzymes play a role in urea synthesis from ammonia (Nelson and Cox, 2002). Briefly, 1) the carbamoyl phosphate is formed from ammonia and bicarbonate (CPS catalyzes this reaction); 2) OTC catalyzes the reaction between ornithine and carbamoyl phosphate forming citrulline; 3) the second group amino from aspartate is added to citrulline by condensation reaction to form arginosuccinate (this reaction occurs in the cytosol and is catalyzed by the ASS); 4) Arginosuccinate is broken down into arginine and fumarate by ASL; and 5) arginine is broken down into urea and ornithine by ARG. Given the importance of understanding protein supplementation, the urea cycle, and the enzymes involved in this process, previous research has been conducted to evaluate these to improve the efficiency of N use (Waldo, 1968; Sun et al., 2016; de Moura et al., 2020). The current study evaluated the 5 enzymes involved in this process, and we observed differences in gene expressions between SUP and non-supplemented animals for CPS, ASL, and ARG. Moreover, we observed a positive linear response for CPS among SUP animals.

Previous studies have demonstrated that CPS is considered a rate-limiting step within the urea cycle (Takagi et al., 2008) because it is responsible for converting ammonia into carbamoyl phosphate (Visek, 1979). Moreover, CPS is the first enzyme in the pathway for urea synthesis, and its activity is an essential step for ammonia detoxification by ruminants (Meijer et al., 1985). Thus, the CPS activity results align with the treatment differences in CP intake between SUP and non-supplemented animals and among SUP animals. In agreement with our results, Takagi et al.(2008) reported increased urea concentration in blood accompanied by increased CPS activity in Holstein calves after weaning (6 weeks of age).

Other researchers have also reported a positive correlation between urea cycle enzymes and urea synthesis rates, CPS and ASL included (Rattenbury et al., 1980). Payne and Morris (1969) also observed greater activity of urea cycle enzymes in sheep fed a high-protein diet than those fed a low-protein diet. Therefore, the greater expression of CPS, ASL, and ARG in the SUP animals than in non-supplemented animals is likely linked to the increased nitrogen intake, leading to greater urea cycle enzyme expression and, consequently, urea production (Castro, 2023).

Surprisingly, differences in gene expression for OTC and ASS between SUP and non-supplemented animals and among SUP animals were not observed. Given the differences observed for other urea cycle enzymes, as cited previously, it is unclear why we observed these results for OTC and ASS. This may suggest that the supplementation level was not extreme enough to alter the expression of these genes involved in the urea cycle. Nonetheless, we emphasize that further research must be carried out with grazing cattle to clarify these results.

CONCLUSIONS

The supplementations of CP to pasture-fed heifers did not affect mammary gland development. However, the results indicate that the mammary gland could remodel itself based on pasture availability (energy and protein) throughout the year. On the other hand, CP supplementation increased the rib eye area and fat thickness. Further, the supplementation directly affected the AMPK expression, where a greater AMPK expression was observed in non-supplemented animals than in SUP animals, thus, confirming the regulatory effects of AMPK on cell growth, which are controlled by the availability of nutrients. Lastly, CP supplementation stimulated the expression of certain urea cycle enzymes (CPS, ASL, and ARG), and a positive linear response to protein supplementation was observed for CPS. Overall, these results indicate that greater CP intake led to increased urea cycle enzymes' expression and promoted greater urea production in the liver.

ACKNOWLEDGMENTS

We are grateful to Minas Gerais State Research Support Foundation (FAPEMIG, Belo Horizonte, Brazil) for funding this study (AUC-00064-16), and the National Council for Scientific and Technological Development (CNPq; Brasilia, Brazil), Higher Education Personnel Improvement Coordination (CAPES), and the National Institute of Science and Technology of Animal Science (INCT-CA, Viçosa, Brazil) for supporting this study. The authors have not stated any conflicts of interest.

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